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(54) Title: PURIFICATION OF PROTEINACEOUS MATERIAL

(57) Abstract

Methods for the purification of proteinaceous materials, such as recombinant glucocerebrosidase (GCS) expressed in an insect cell culture that had been previously infected with a baculovirus carrying GCS-encoding nucleic acid, are disclosed. According to one embodiment, the medium is first clarified by being passed through a hollowfiber cartridge, then bacteria is filtered out. The filtered media is then concentrated with a cation exchange column, followed by hydrophobic interaction chromatography (HIC) purification. In alternative embodiments, the eluate from the cation exchange column and/or the HIC column is passed through an anion exchange column for removal of residual DNA, lipids and endotoxins. The purification methods of the present invention are quicker, cheaper, and capable of handling greater quantities and greater starting purities of conditioned media than processes previously utilized. The various methods of the present invention are also capable of providing commercial quantities of purified GCS suitable for therapeutic uses. Purified forms of GCS are also disclosed.

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PURIFICATION OF PROTEINACEOUS MATERIAL

The present invention is directed to methods for the purification of proteinaceous material, such as the enzyme glucocerebrosidase (GCS), from various expression media and the contaminating or other cellular proteins co-expressed in the media. The present invention is also directed to proteins purified by the methods.

Background of the Invention

Gaucher's disease is a genetic disorder which results in the accumulation of large quantities of the glycolipid glucocerebroside in the lysosomes of reticuloendothelial cells. The cause of Gaucher's disease has been found to be a deficiency in the lysosomal enzyme glucocerebrosidase ("GCS"), which hydrolyzes glucocerebroside. Clinical manifestations of the disease include splenomegaly, hepatomegaly, skeletal disorders, thrombocytopenia and anemia. It has been estimated that there are about 20,000 cases of this genetic disease in the U.S. alone. It has been found that there is a high correlation between the severity of the clinical manifestations of the disease and the degree of attenuation of glucocerebrosidase activity.

One method of treating Gaucher's disease includes the administration of an enzymatically active amount of recombinant GCS to the patient which results in the hydrolyzation of the glucocerebroside. Two previously disclosed sources of GCS were placental tissue and genetically manipulated GCS-encoding nucleic acid which was inserted into various cells such as insect cells,

yeast cells or mammalian cells. Those skilled in the art will appreciate that reliance upon human placental tissue has the inherent disadvantage of limited availability and the risk of viral contamination. Previously disclosed methods of forming recombinant GCS have required difficult, multi-step purification processes in order to obtain even minute quantities of GCS. Some purification processes for recombinant GCS have also required the use of materials which are inappropriate for therapeutic use, therefore, requiring additional steps to remove these materials before administering the "purified" enzyme to humans. Such processes also often include steps that are unsuitable for commercial-scale production of purified enzyme.

It is therefore desirable to provide a purification method which can be carried out on a large scale, commercial basis with improved cost effectiveness.

It would also be desirable to provide a purification method which does not employ materials unsuitable for therapeutic purposes which must later be removed. It is also desirable to provide a purified form of GCS having therapeutically acceptable levels of residual endotoxins, DNA and lipids.

Summary of the Invention

Embodiments of the present invention comprise the purification of proteinaceous materials harvested from various media, such as an insect cell culture that had been expressing or secreting a proteinaceous material, for example GCS, by infection with a recombinant baculovirus.

According to one embodiment of the present invention, a conditioned medium containing a secreted protein is clarified by first passing the media through a hollowfiber cartridge, with collection through a filter. In this method, the media is concentrated through a cation exchange column which captures the desired protein, followed by further purification by hydrophobic interaction chromatography (HIC).

According to another embodiment, the eluate from the cation exchange column, which comprises the enzyme glucocerebrosidase (GCS), is passed through an anion exchange column either prior to or after purification on an HIC column to make the GCS therapeutically acceptable.

According to one preferred embodiment of the present invention, the HIC purification comprises loading the cation exchange eluate containing GCS onto a butyl-substituted HIC column. After washing the column with a suitable washing buffer, such as ammonium sulfate, the column is washed with a first mixture of absolute (anhydrous) ethyl alcohol and sodium acetate buffer, followed by a second washing containing a higher concentration of ethyl alcohol, preferably at a higher pH, which serves to elute the enzyme from the column. One advantage of this method of the present invention is that the protein, whether pretreated with anion or cation exchange resins or even with no pretreatment, is acceptable for loading directly onto the HIC column. A second advantage is the use of step elution techniques which have distinct advantages over gradient elution especially on large commercial scales.

The purification methods of the present invention are quicker, cheaper, and capable of handling greater quantities and higher starting purities of conditioned media then processes previously utilized. The various methods of the present invention are capable of providing commercial scale quantities of purified GCS suitable for therapeutic uses. By eliminating the use of substances which are unsuitable for therapeutic uses, the need for additional steps to remove the substances is eliminated. The present invention is also capable of reducing the levels of endotoxins, DNA and lipids to therapeutically acceptable levels.

The purified recombinant GCS made in accordance with the methods described herein are enzymatically active on synthetic substrates such as 4-MUG (4-methylumbelliferyl-α-D-glucopyranoside), as well as on native glucocerebroside.

Detailed Description

The various embodiments of the present invention provide novel methods for purifying proteinaceous materials, such as the enzyme glucocerebrosidase. The starting materials may comprise materials which have been prepared in a culture medium or extracted from natural sources. The starting materials of these methods may include proteinaceous material that is expressed by eukaryotic or prokaryotic cells, such as insect, mammalian or yeast cells, by inserting an appropriate encoding nucleic acid into the cell under conditions suitable for expression. The nucleic acid is typically placed within a vector, such as a plasmid or virus, for expression in the target cell.

The starting material of one preferred embodiment comprises a baculovirus expression system which secretes GCS, such as those described in PCT patent publication International Publication Number WO 89/05850 to Ginns et al. Alternatively, the recombinantly expressed enzyme disclosed in PCT patent publications International Publication Numbers WO 90/07573 to Rasmussen and WO 92/13067 to Hayes may also be purified according to the methods described herein. GCS extracted from human placental tissue as disclosed in U.S. Patent No. 3,910,822 to Pentchev et al. may also be used as starting material. Each of these publications is hereby incorporated by reference.

One particularly preferred starting material is prepared by inserting a virus carrying GCS-encoding nucleic acid into an insect cell culture under conditions suitable for expression within those cells. For example, the insect cells may be cultured in a commercially available medium, such as SF900 II sold by Gibco Corp. of Grand Island, New York. The cells are preferably cultured to log phase, e.g. about 4-6 X 10^6 or more cells per ml of medium prior to infection.

In order to further increase the efficiency of the purification methods of the present invention, a 100% cell culture media exchange is also preferably performed. Those skilled in the art will appreciate that by replacing nutrient depleted media with nutrient replenished media, the cell concentration will be increased and the cells will tend to stay alive longer after infection.

The cell culture medium is then infected with a virus carrying the GCS gene and maintained at conditions suitable for expression of the GCS. For example, the media can be maintained at a temperature of about 28°C for about 3-5 days before harvesting.

One embodiment of the purification process of the present invention comprises the steps of:

1) Clarifying the conditioned media by hollowfiber microfiltration to remove any cells, cell debris, and also preferably filtering to remove incidental bacteria;

2) increasing the enzyme/liquid-volume concentration with an enzyme capturing cation exchange resin;

3) removing other therapeutically unacceptable compounds such as residual endotoxins, DNA and lipids, utilizing an anion exchange resin; and

4) subjecting the resulting solution to hydrophobic interaction chromatography (HIC) purification with a "step" elution.

While various methods of clarifying the conditioned media may be utilized, the following is one preferred method. The conditioned medium is passed through a hollowfiber cartridge, preferably having a pore size of about 0.2 to about 0.45μ, most preferably about 0.35μ. The use of a hollowfiber cartridge is a preferred method for separating the conditioned medium into a first clarified-medium fraction containing GCS and a second cellular-debris fraction containing larger particles. For example, a suitable hollowfiber cartridge having a pore size of 0.35 microns can be obtained from Sepracor,

Marlborough, Massachusetts. Hollowfiber microfiltration is preferred over previously disclosed steps of centrifugation since the hollowfiber microfiltration is more readily adaptable to large-scale production.

The clarified-medium fraction obtained from the hollowfiber microfiltration is then preferably filtered to remove incidental bacteria. For example, a 0.22μ filter is suitable to remove bacteria that may get into the solution during the processing. This second filtration step is particularly advantageous if the conditioned medium is going to be stored as it will prevent bacterial growth and stabilize the enzyme. At this point, the conditioned medium can be safely stored for periods of up to at least 4 weeks at a temperature of about 2 - 8°C.

Those skilled in the art will appreciate that a hollowfiber cartridge can accommodate larger volumes of material over a given time period than could a centrifuge. If a relatively small amount of conditioned media was being clarified in this step, the media could alternatively be spun in a centrifuge, for example at about 10,000 - 12,000 g's for about 20 minutes and then filtered, e.g. through a 0.22μ polysulfone filter. Somewhat smaller or intermediate volumes of media can also be clarified by using a sandy material such as Celite, available from Sigma Chemical, St. Louis, Mo.

The clarified medium is first acidified to a pH of about 4.5-6, preferably about 5.0 to about 5.5, most preferably about 5.1 $\pm$ 0.1 with 25 percent by volume acetic acid. The acidified medium is then loaded into an enzyme-capturing cation exchange column, for example a

sulfopropyl column. Those skilled in the art will also appreciate that the charge of the protein differs with pH. Thus, it is feasible that other enzyme-capturing cation exchange resins could be successfully utilized at this or another pH. A pH of about 5.1, ± 0.1 has been found to be desirable when used with a sulfopropyl column. As used herein, the term "sulfopropyl" column refers to a cation exchange column having sulfopropyl functional groups attached to a polymer or resin. These sulfopropyl functional groups can be attached to any of a variety of polymers or resins such as a methacryl polymer. The resin sold under the name "SP Trisacryl PLUS M" marketed by IBF of Garenne, France, for Sepracor, has been shown to work well. Alternatively, Poros HS available from Perceptive Biosystems, Cambridge, MA is also suitable.

The enzyme-capturing cation exchange column is then washed with a suitable washing buffer, for example an acetate buffer, citrate buffer or phosphate buffer, at a concentration of up to about 150 mM, to remove contaminating proteins while leaving GCS on the resin. One preferred washing buffer comprises an acetate buffer having a pH of 5.1 ± 0.1. This wash is preferably continued until substantially no further proteins are being washed from the column as indicated by a UV monitor at the column exit. Those skilled in the art will appreciate that a UV monitor set at a wavelength of 280 nanometers (280A) will indicate the presence of proteins and can therefore be readily used to determine when a wash step has been completed. The absorbance level selected for the "baseline" is as low as possible and is normally indicated by an absorbance reading which does not change over a 3 minute period. The enzyme is then

eluted with a solution of ammonium sulfate, preferably in the same buffer used in the wash. For example, about 50 - 150mM acetate buffer containing about 250mM to 500mM ammonium sulfate and, optionally, a small amount of pluronic is suitable. Preferably, a suitable salt concentration is at least 300mM and is capable of removing substantially all of the GCS enzyme from the cation exchange column. This volume reduction step serves to concentrate the enzyme solution. This step can advantageously reduce the liquid volume of the enzyme solution at least 20 fold, for example from about 100 liters to about 5 liters, increasing the enzyme concentration from about 5 mg/ltr to about 100 mg/ltr, greatly reducing the processing time. This is one of the significant advantages of the present invention and is particularly desirable when viewed from a commercial-production perspective.

After the volume reduction step using the enzyme-capturing cation exchange column, the eluate can advantageously be subjected directly to the hydrophobic interaction chromatography purification. The positively charged protein leaves the cation exchange column with a relatively high salt content which is suitable for HIC purification without requiring any intermediate modification.

Alternatively, residual endotoxins, DNA and lipids may be reduced to therapeutically acceptable levels at this point in the process using a suitable anion exchange resin. A clean, sanitized anion exchange resin is used, capable of binding DNA, lipids and endotoxins but not the GCS at the pH of the GCS solution. DNA, lipids or endotoxins present will bind to the anion

exchange resin, with the GCS remaining in solution. Suitable anion exchange chromatography resins are capable of binding DNA, lipids and endotoxins, but not GCS, at a pH less than about 7.0. These resins can be readily identified by evaluation with standardized GCS solutions having the required pH and osmolality. The GCS solution should have a known GCS concentration as well as a known endotoxin and lipid concentration. The endotoxin should be a commercially available endotoxin standard. The lipids may be a mixture of lipids commonly found in the culture media utilized in the process.

One manner of removing these unwanted substances comprises first diluting the fraction which was eluted from the sulfopropyl (SP) column, one fold with a buffer. For example, a sodium acetate buffer comprising about 10 to 50 mM sodium acetate having a pH of about 6 to 6.2 and, optionally about 0.025 to 0.5 percent by weight of pluronic. This fraction is then loaded onto an anion exchange column, for example a DEAE column. The anion exchange column is then washed with a low ionic strength buffer, such as ammonium sulfate, with a concentration of about 20 to 70 mM, preferably about 50 mM ± 5 mM until the optical density as measured by the UV monitor indicates that substantially all non-binding proteins have passed through the column. GCS flows through the column while DNA, endotoxins and lipids bind to the anion exchange column. The GCS solution may then be further purified by HIC.

The hydrophobic interaction chromatography purification comprises loading the SP eluate or anion exchange flow-through onto a column containing a hydrophobic, butyl-substituted resin/polymer, for

example, Toso-butyl available from Toso-Haas, or any hydrophobic butyl- or phenyl-substituted resin/polymer suitable for HIC. The column is then washed, for example with a wash buffer containing about 0 to 60mM ammonium sulfate, for as long as needed as indicated by the UV monitor. The column is then washed with a first buffer solution of absolute ethyl alcohol and sodium acetate buffer having a pH of about 5 to 6.5, preferably about 6.0 to 6.4. It is well known that GCS is not stable in the alkaline pH range and such pH should be avoided. This solution contains up to about 25% vol/vol absolute ethyl alcohol, preferably about 18 to about 24% ethyl alcohol and about 50 to 150 mM sodium acetate buffer, most preferably about 96 to 100 mM. This washing is preferably continued with at least 20 column volumes until the optical density reaches baseline.

When the baseline is reached, this first washing with ethyl alcohol is stopped and an elution buffer solution is started containing a higher percentage, preferably at least about 5 percent more, and preferably at least about 10% more ethyl alcohol than in the first ethyl alcohol wash. The second ethyl alcohol wash solution preferably comprises about 30 to 40 percent vol/vol absolute ethyl alcohol, most preferably about 35 to 38 percent, about 100 to 150 mM sodium acetate buffer, most preferably about 140 to 145 mM and a pH of about 5 to 6.7, most preferably about 6 to 6.5. The eluted GCS enzyme is active and therapeutically pure. Isopropyl alcohol may be substituted for ethyl alcohol if desired.

If the eluate from the cation exchange column had not already been passed through an anion exchange column as described above, this step is preferably

performed with the HIC eluate at this point. The conditions used for removing DNA, endotoxins and lipids at this point are the same as those described above.

Optionally, the purified GCS can be collected in a tube containing about 40 to 60 mM sodium acetate buffer, preferably about 50 mM at a pH of about 4.9 to 5.1, preferably about 5, and about 0.02 to 0.03 percent by volume Tween 80, most preferably about 0.025 percent by vol. Tween 80.

Another aspect of the present invention comprises purified forms of GCS. One embodiment is a clear, colorless solution having a protein concentration of 0.5 - 2.0 mg/ml as estimated by BCA assay with BSA as standards. The endotoxin level is less than 10 EU/mg protein, preferably less than 5 EU/mg, most preferably less than 3 EU/mg. The specific activity, as estimated per 4-MUG assay is at least 30 International Units/mg protein, preferably at least 40 International Units/mg protein. The purity is greater than 98% with no single impurity more than 1%. The purified forms of GCS obtained by the various methods of the present invention run as single bands on SDS gel. The apparent molecular weight as determined by the single band using molecular weight standards is 59 - 60 kD. The purified forms are enzymatically active against synthetic substrates such as 4-MUG and native substrates such as glucocerebroside.

EXAMPLE 1

The starting material of this example comprised cultured Trichoplusia ni cells sold under the name "HIGH-FIVE" cabbage looper cell line BTI TN5B1-4 by Invitrogen

Corp. of San Diego, CA, which had been infected with baculovirus carrying GCS-encoding nucleic acid described in PCT WO 89/05850 and incubated. The cell culture was loaded directly onto a Sepracor hollowfiber cartridge having a pore size of 0.35μ. The GCS enzyme-containing fraction (conditioned medium) was then filtered by passing it through a 0.22μ filter into a sterile bag. These steps advantageously removed insect cells, cell debris and bacteria from the GCS fraction.

The conditioned medium was then acidified with acetic acid to a pH of 5.1 $\pm$ 0.1 in preparation for loading onto a sulfopropyl resin, SP Trisacryl PLUS M, from IBF. The packing buffer and equilibration buffer both comprised 100 mM acetate buffer having a pH of 5.1 $\pm$ 0.1. Alternatively, a citrate buffer could be substituted. The resin utilized had a binding capacity of about 200 ml of conditioned medium per ml of resin at a flow rate of about one-third column volume to about one-half column volume per minute as estimated by 4-MUG assay. The column was packed with the resin and packing buffer and then equilibrated with the same buffer. 200 liters of conditioned medium per liter of resin (calculated with a total protein content of conditioned medium being ca 3 mg/ml) was loaded into the column. The column which had a height of 5.2 centimeters and a diameter of 18 centimeters was then washed with 10 column volumes of 100 mM acetate buffer having a pH of 5.1 $\pm$ 0.1 at a flow rate of 0.5 column volumes(cv)/min (linear velocity = 156 cm/hr). Alternatively the column could have been washed with this acetate buffer until the optical density returned to baseline as indicated on a UV monitor connected to the column discharge. The GCS was then eluted with an elution buffer containing 100 mM

acetate buffer, a pH of 5.1 ± 0.1 containing 250 mM ammonium sulfate. This eluate is stable at 4°C for a minimum of three weeks.

The product recovery from this volume reduction step was found to be 100 percent by enzyme activity. The column can also be regenerated using a regeneration buffer having ten column volumes of 1 M NaCl, 10 cv of 0.1 N NaOH, followed by 20% by volume isopropyl alcohol (IPA) until the pH is less than 7.5.

A butyl-substituted hydrophobic interaction chromatography resin, TosoHaas Butyl 650C, was then packed into a column using a packing buffer with 150 mM acetate buffer, pH 6.0 ± 0.1 containing 40% by volume ethanol. The column was equilibrated with 10 cv of 50 mM ammonium sulfate in water having a pH of about 5.5. The eluate from the SP Trisacryl column containing the GCS was then loaded directed onto the equilibrated butyl column. The butyl column is then washed with 10 cv of 50 mM ammonium sulfate followed by 10 cv of 24% by volume ethanol in 100 mM acetate buffer having a pH of 6.0 ± 0.1. The GCS was then eluted with an elution buffer having 150 mM acetate buffer, pH 6.0 ± 0.1 containing 36% by volume ethanol, final pH 6.5. The eluted enzyme was found to run as a single band in polyacrylamide gel electrophoresis using a buffer that contains sodium dodecyl lauryl sulfate (SDS-PAGE). This enzyme is stable in this elution buffer for a minimum of one week and can be stored for longer periods in solutions containing 20% by volume glycerol and/or a non-ionic detergent, for example about 0.025% by volume Tween 80.

EXAMPLE 2

This example utilized the same starting material and same procedures used in Example 1 with the addition of a step to reduce the levels of DNA, lipids and endotoxins to therapeutically acceptable levels. This additional step was performed to the cation exchange eluate prior to the HIC purification by diluting the fraction which was eluted from the sulfopropyl (SP) column one fold with a sodium acetate buffer comprising 20 mM sodium acetate having a pH of about 6 ± 0.1 and 0.025 percent by wt. pluronic. This fraction was then loaded onto a DEAE anion exchange column. The anion exchange column was then washed with an ammonium sulfate buffer with a concentration of 50 mM until the optical density, as measured by the UV monitor, indicated that substantially all non-binding proteins have passed through the column. This step allowed the rGCS to flow through the column while DNA and endotoxins are bound to the resin. The rGCS material flowing through the column was then loaded onto the butyl-substituted hydrophobic interaction chromatography resin and processed as in Example 1 to obtain the purified rGCS.

EXAMPLE 3

In this example, particles are removed from the culture by hollowfiber microfiltration and the particle free material is directly loaded onto a hydrophobic media butyl column made by Toso-Haas referenced above. The column is then washed with 10 column volumes of 50 mM ammonium sulfate followed by 24% by volume of ethanol in 90 - 100 mM acetate buffer having a pH of 6.25 ± 0.1. The GCS is then eluted with an elution buffer having 145-

155 mM acetate buffer, pH 6.5 ± 0.1, containing 36% by volume ethanol. The eluted enzyme runs as a single band in polyacrylamide gel electrophoresis using a buffer containing sodium lauryl sulfate (SDS-PAGE).

From the present description, those skilled in the art will appreciate that the present invention can be readily scaled up to commercial production. The processes of the present invention also advantageously provide a purified GCS that is in a therapeutically-acceptable form.

WE CLAIM:

1. A method of purifying a proteinaceous material comprising the steps of:

loading said proteinaceous material onto a hydrophobic, butyl-substituted chromatography column;

washing said chromatography column with a wash buffer comprising ethanol in an amount not greater than about 25% by volume and having a pH of below 7; and

eluting protein from said chromatography column with an elution buffer comprising at least 5% by volume more ethanol than said wash buffer and having a pH of less than 7.

2. A method according to claim 1 wherein said step of loading proteinaceous material onto said hydrophobic chromatography column comprises loading a protein which is hydrophobic and has a positive charge at a pH of 4.5 - 6.8.

3. A method according to claim 1 wherein said step of loading proteinaceous material onto said hydrophobic chromatography column comprises loading glucocerebrosidase onto said column.

4. A method according to claim 1 further comprising the steps of:

providing a cell culture medium which has been infected with a virus carrying GCS-encoding nucleic acid; and

clarifying said cell culture medium, and wherein said clarified medium is said proteinaceous material loaded onto said column.

5. A method according to claim 4 further comprising the step of filtering said clarified medium before loading said clarified medium onto said column.

6. A method according to claim 5 wherein said step of filtering said clarified medium comprises passing said clarified medium through a filter having a pore size of about 0.2 - 0.45 μ before loading said clarified medium onto said column.

7. A method according to claim 4 further comprising the steps of:

acidifying said clarified medium to a pH of about 4.5 - 6; and

increasing the concentration of said clarified-medium fraction by loading said clarified-medium fraction into an enzyme-capturing cation exchange column;

washing said enzyme-capturing cation exchange column with a first buffer having a pH of about 5 - 6; and

then eluting glucocerebrosidase from said enzyme-capturing cation exchange column with a second buffer comprising ammonium sulfate.

8. A method according to claim 7 wherein said second buffer comprises 50 - 150 mM acetate buffer and at least 250 mM ammonium sulfate.

9. A method according to claim 8 wherein said first buffer comprises an acetate buffer having a pH of 5.1 ± 0.1.

10. A method according to claim 7 wherein the eluate from said enzyme-capturing cation exchange column is passed through an anion exchange column prior to loading onto said chromatography column.

11. A method according to claim 10 wherein said step of passing the eluate from said enzyme-capturing cation exchange column through an anion exchange column comprises:

loading the eluate from said enzyme-capturing cation exchange column into a DEAE anion exchange column; and

washing said column with an ammonium sulfate solution.

12. A method according to claim 7 wherein said glucocerebrosidase eluted from said chromatography column is subsequently passed through an anion exchange column.

13. A method according to claim 7 wherein said enzyme-capturing cation exchange column comprises a resin comprising sulfopropyl functional groups.

14. A method according to claim 7 wherein said wash buffer comprises about 18% - 24% by volume ethanol.

15. A method according to claim 14 wherein said wash buffer comprises a pH of 6.0 $\pm$ 0.1.

16. A method according to claim 14 wherein said elution buffer comprises about 30% - 40% by volume ethanol.

17. A method according to claim 14 wherein said elution buffer comprises about 35% - 38% by volume ethanol.

18. A purified protein obtained by the method of claim 7.

19. A purified protein obtained by the method of claim 1.

20. A process for purifying a cell culture medium comprising GCS comprising the steps of:

providing a cell culture medium which is forming GCS;

clarifying said cell culture medium to form a clarified medium fraction;

acidifying said clarified-medium fraction to a pH of about 4.5 - 6;

increasing the glucocerebrosidase/liquid-volume concentration of said clarified-medium fraction; and

loading said GCS-containing fraction onto a hydrophobic, butyl-substituted chromatography column, washing said chromatography column with a wash buffer comprising ethanol in an amount not greater than about 25% by volume, and then eluting glucocerebrosidase from said chromatography column with an elution buffer comprising at least 5% by volume more ethanol than said wash buffer.

21. A process according to claim 20 wherein said clarifying step comprises passing the culture medium through a hollow fiber cartridge comprising fibers having a pore size of about 0.2 - 0.45μ to separate said

acidified culture medium into a cellular-debris fraction and a clarified-medium fraction comprising glucocerebrosidase.

22. A process according to claim 20 wherein said concentration increasing step comprises:

loading said clarified-medium fraction into an enzyme-capturing cation exchange column,

washing said enzyme-capturing cation exchange column with a first buffer having a pH of about 4.5 - 6, and

eluting glucocerebrosidase from said enzyme-capturing cation exchange column with a second buffer comprising ammonium sulfate.

23. A process according to claim 22 wherein the eluate from said enzyme-capturing cation exchange column is passed through an anion exchange column prior to loading onto said chromatography column.

24. A method according to claim 20 wherein said glucocerebrosidase eluted from said chromatography column is subsequently passed through an anion exchange column.

25. A process according to claim 20 wherein said step of providing a cell culture medium comprises providing a culture medium which has been infected with a virus carrying GCS-encoding nucleic acid.

26. A process according to claim 25 wherein said step of providing a cell culture medium comprises providing an insect cell culture medium which has been infected with a baculovirus carrying GCS-encoding nucleic

acid.

27. A process according to claim 20 wherein said wash buffer comprises about 18% - 24% by volume ethanol.

28. A process according to claim 20 wherein said elution buffer comprises about 30% - 40% by volume ethanol.

29. A purified form of GCS comprising at least 98% GCS.

30. A purified form of GCS according to claim 29 wherein any single impurity is present in an amount less than 1%.

31. A purified form of GCS according to claim 29 having a specific activity of at least 30 IU/mg.

32. A purified form of GCS according to claim 31 having a specific activity of at least 40 IU/mg.

33. A purified form of GCS according to claim 32 having an endotoxin level of less than 10 EU/mg protein.

34. A purified form of GCS according to claim 33 having an endotoxin level of less than 3 EU/mg protein.

35. A purified form of GCS according to claim 29 having a protein concentration of about 0.5 - 2.0 mg/ml.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12395

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 3/12, 3/18, 3/20, 3/22, 3/26, 3/28; C12N 9/24
US CL :530/ 412, 414, 415, 416, 417; 435/69.1, 200

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/ 412, 414, 415, 416, 417; 435/69.1, 200

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Online, Medline, APS, World Patents Index

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/07573 (Rasmussen et al) 12 July 1990, see pages 14-16.	1-35
Y	US, A, 5,089,605 (Profy et al) 18 February 1992, see column 5, lines 40-43.	5-6,18-19,21,29-35
Y	US, A, 3,910,822 (Pentchev et al) 07 October 1975, see column 2 through column 3.	7 - 9 , 1 4 - 19,20,22,25-35
Y	BioTechniques, Volume 1, issued November 1983, G. Sofer et al, "Designing an Optimal Chromatographic Purification Scheme for Proteins", pages 198-203, see entire document.	10-13,18-19,23-24,29-35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search
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Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12395

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Pharmacia, "Ion Exchange Chromatography, Principles and Methods", published 1983 by Rahms i Lund (Sweden) pages 1-38, see entire document.	10-13,18-19,23-24,29-35